Modulation of planaria regeneration by Resolvin D1 and the omega-3 fatty acid precursor 17-hydroxy docosahexaenoic acid

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SUMMARY
Omega-3 fatty acids (FA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) have long been consumed as medical supplements for their far-reaching health benefits, ranging from alleviating asthma symptoms to mitigating kidney and inflammatory diseases like colitis and diabetes. These fatty acids produce a variety of lipid mediator metabolites including maresins, protectins, and resolvins, and are essential for aiding the resolution pathways in inflammation. However, their roles in regenerative responses are relatively unknown. We decided to investigate the effect of Resolvin D1 (RvD1) in Dugesia dorotocephala regeneration, as RvD1 is the most widely studied lipid mediator. We found that regenerating planaria released significantly higher amounts of RvD1 in water than control and that D. dorotocephala could synthesize their own RvD1 from the omega-3 FA precursor 17-hydroxy docosahexaenoic acid (17-HDHA). We also observed that both RvD1 and 17-HDHA enhanced regeneration responses in planaria by using eye scoring and a modified cell metabolism assay (MTT). We conclude that planarians may utilize similar biosynthetic pathways to mammals in producing resolvins and that resolvins hold immense potential in enhancing regenerative responses in tissues.

INTRODUCTION
Omega-3 fatty acids (FA) are essential for regulating many homeostatic bodily functions such as the contraction of arterial walls, hormonal regulation, controlling anti-inflammatory responses in chronic ailments such as arthritis and asthma, and modulating many homeostatic responses (1). Humans rely on procuring these fatty acids from their diets because humans are unable to synthesize these omega-3 fatty acids (2).

Chronic deregulated inflammation is the causal factor for some of the most common human diseases such as asthma, colitis, arthritis, diabetes. In the last decade, numerous mediators formed through the metabolism of omega-3 fatty acids have been implicated in the resolution of inflammation (3). Maresins are a specific type of lipid mediator formed through the metabolism of the DHA (docosahexaenoic acid) branch of omega-3 fatty acids. In 2009, it was found that maresins are a class of potent, anti-inflammatory mediators that enhance the resolution phase of inflammation in a mouse model of peritonitis (4). This was the first time that maresins and other similarly structured lipid mediators were shown to play a key role in orchestrating tissue homeostasis, inflammation resolution, wound healing, and host defense (4). A study observing the effects of maresins on regeneration in planaria found that maresins enhanced or sped up regeneration in planarians after surgical incision and reduced “neuropathic pain” in mice (5). In planaria specifically, the study used immunofluorescence to quantify the amount of regeneration occurring at the severed ends of the cut planaria (5). This is the only published evidence of a role for omega-3 fatty acid-derived lipid mediator in planarian regeneration.

RvD1 and its precursor 17-hydroxy docosahexaenoic acid (17-HDHA) are a sister branch to the maresin lipid mediator and are also derived from DHA (6). A study in 2013 found that the external administration of resolvins does not disrupt homeostasis. Rather, it resolves acute inflammatory pain and chronic arthritis (7). In other words, in addition to inhibiting or blocking inflammation, resolvins incite the resolution of inflammation and promote the return to homeostasis (3). To our knowledge, no published study observing the effects of RvD1 and its precursor, 17-HDHA, on planaria exists.

Planaria are a unique model for studying regeneration because they are able to regenerate fully even when large portions of their body are removed, and they fully regenerate within 7 to 21 days. Planaria regenerate new tissue around a wound site, form blastema through epimorphosis and repair existing tissue through morphallaxis (8). New technologies, such as RNA interference, have been used to study the planarian stem cells and genome on a molecular level, which has revealed profound similarities between several parts of the planarian genome and genes that cause diseases in humans (9). Planaria’s rare regenerative abilities are dependent on their large population of somatic stem cells. Both planaria and vertebrates share the functionally conserved Wnt/β-catenin and BMP signal transduction pathways for axis polarity, presenting an opportunity to research pluripotent stem cells in planaria to better understand mammalian disease and development and to better analyze relevant molecular processes in humans (9). Further research on planarian’s highly-flexible central nervous system (CNS) may also reveal the machinery involved in activating axon regeneration and circuitry reformation in planaria, which may be critical in understanding activation of regeneration in mammals (9).

In this study, we measured planarian’s regenerative responses with supplementation of RvD1 and 17-HDHA using an eye scoring index and a modified MTT assay. Additionally,
we wanted to investigate if planarians could synthesize RvD1 from 17-HDHA. In this study, we show that planarians have the biosynthetic machinery to produce RvD1 from 17-HDHA. and that planaria might be able to respond to RvD1 if given exogenously.

RESULTS

Tissue and water RvD1 levels in regenerating planaria

We first investigated if Dugesia dorotocephala can biosynthesize RvD1 from the omega-3 fatty acid precursor 17-HDHA. Secondly, we also looked at short-term and long-term RvD1 production by D. dorotocephala incubated with 17-HDHA. Concentrations for RvD1 were chosen between .001 nM and 100 nM because effects of RvD1 are typically observed at nanomolar concentrations (1). Since RvD1 is formed from 17-HDHA, we used a 10 to 100 times higher concentration for 17-HDHA.

RvD1 from water samples harvested from planarian environment exhibited a significant increase in regenerating planaria compared to whole planaria incubated in 17-HDHA for 3.5 days (student’s t-test, p=0.0002). While both control groups (whole & cut planaria) exhibited the same baseline RvD1 level of 0.0975 pg/mL (SEM: 0.002), 17-HDHA supplementation resulted in an increase in RvD1 production of up to 2.900 pg/mL (SEM +/- 2.185) in whole planaria and up to 14.28 pg/mL (SEM +/- 1.789) in regenerating planaria (Figure 2).

Figure 1. Tissue RvD1 levels in whole versus regenerating planaria. Planaria lysates in PBS were analyzed 3.5 days post-surgery. Each dot represents 2 planaria. Regenerating group represents 2 planaria (2 heads and 2 tails). Data is normalized to µg proteins per sample. Mean ± SEM. Student’s t- test was performed between control (Ctrl) and experimental groups. *p<=0.05.

Figure 2. Water RvD1 levels in whole versus regenerating planaria. Water RvD1 levels in whole vs. regenerating planaria. RvD1 levels in water (planaria habitat, 500 µl) were analyzed 3.5 days post-surgery. Each dot represents 2 planaria. Regenerating group represents 2 planaria (2 heads and 2 tails). Data is mean ± SEM. Student’s t- test was performed between Ctrl and experimental groups. *p<=0.05.

RvD1 dose response measured through planarian eye scoring index.

An eye scoring index was created to categorize planarian regeneration into five stages based on the formation of photoreceptors in planaria tails post-surgery (Figure 4). Previous studies showed that optic regrowth and functional recovery were consistent despite injury type or differences in metabolic rate due to starvation (10). Therefore, eye scoring was utilized as an independent surrogate marker of regeneration, and we additionally scored the planarians’ eyes that included several stages of complete eye formation.

The eye scoring index was developed to study the planarians’ regeneration response to three dosages of RvD1 (0.01 nM, 0.1 nM, and 1.0 nM) at both a shorter time point (2 days) and a longer time point (8 days). After 2 days, the concentration of RvD1 in the water of planaria incubated with 1 µM 17-HDHA was 1.45 pg/mL (SEM +/- 1.150), while planaria incubated in 10 µM 17-HDHA did not produce detectable RvD1. A significant increase in RvD1 production was observed, however, after 8 days of incubation. Planaria incubated in 1 µM of 17-HDHA produced 45.53 pg/mL (SEM +/- 3.535) of RvD1 (student’s t-test, p<0.05), while planaria incubated in 10 µM of 17-HDHA produced 56.57 pg/mL (SEM +/- 11.63) of RvD1 (Figure 3).

Figure 3. 17-HDHA time and dose response in planarian RvD1 production

We next studied the effect of increasing the 17-HDHA dose by 10-fold (10000 nM or 10 µM) and measured RvD1 production in water at both a shorter time point (2 days) and a longer time point (8 days). After 2 days, the concentration of RvD1 in the water of planaria incubated with 1 uM 17-HDHA was 1.45 pg/mL (SEM +/- 1.150), while planaria incubated in 10 µM of 17-HDHA did not produce detectable RvD1. A significant increase in RvD1 production was observed, however, after 8 days of incubation. Planaria incubated in 1 µM of 17-HDHA produced 45.53 pg/mL (SEM +/- 3.535) of RvD1 (student’s t-test, p<0.05), while planaria incubated in 10 µM of 17-HDHA produced 56.57 pg/mL (SEM +/- 11.63) of RvD1 (Figure 3).
days) and a longer time point (8 days). After 2 days, the eye score of planaria incubated in 0.01 nM of RvD1 was 0.3333 (SEM +/- 0.3333), while planaria incubated in 0.1 nM and 1 nM of RvD1 did not show detectable eye formation. After 8 days of incubation, increased development of photoreceptors in planaria tails were observed. Planaria incubated in 0.01 nM of RvD1 scored on average 1.750 (SEM +/- 0.3660), whereas planaria incubated in 0.1 nM and 1 nM of RvD1 had a mean score of 2.143 (SEM +/- 0.4592) and 1.429 (SEM +/- 0.4286), respectively, on the eye scoring index (Figure 5).

Comparison of planaria response to 17-HDHA and RvD1 using planarian eye scoring index

To measure the reliance of regeneration on type of lipid mediator (17-HDHA or RvD1) and the dose dependency of each drug, an experiment was conducted using the eye scoring index on the regeneration of planaria after incubation in control or 17-HDHA or RvD1 for 4 days.

The planarian eye scoring index was used to compare planarians’ regeneration response to 1 µM of 17-HDHA and two dosages of RvD1 (0.01 nM and 0.1 nM). After 3.5 days of incubation, the eye score of planaria incubated in 1 µM of 17-HDHA was 2.143 (SEM +/- 0.1353), while the eye scores of planaria incubated in 0.01 nM and 0.1 nM of RvD1 were 2.167 (SEM +/- 0.2161) and 2.938 (SEM +/- 0.1133), respectively, on the eye scoring index (Figure 5). All three eye scores were greater than the eye score of the control group (ethanol-supplemented planaria), which was 2.083 (SEM +/- 0.2117) on the planarian eye scoring index.

Using a modification of the MTT assay to quantify planarian regeneration

The planarian eye scoring index provides a semi-quantitative measure of planarian regeneration. We next investigated whether a modification of the MTT assay could be used to analyze planarian regeneration in a quantitative way. The MTT Assay is a cell metabolism assay that uses the crystallization of the violet MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to measure cell viability. When read through a plate reader, a higher optical density (OD) correlates to a higher concentration of violet colored formazan crystals, indicating greater cell proliferation.

The MTT assay was first used to study whole planaria of different sizes (small, medium, and large). After 4 hours of incubation in MTT, whole planaria demonstrated a mean increase of 0.2768 in optical density, compared to control. The optical densities of small, medium, and large planaria were 0.534, 0.561, and 0.592, respectively (Figure 6D).

We then hypothesized that the presence of more regenerating surfaces would result in more formazan crystal formation, producing greater optical densities. The MTT assay was used to analyze various quantities of planaria stubs (laterally-cut segments of the planarian body with two regenerating surfaces) (Figure 6A-C). After 4 hours of incubation in MTT, regenerating planaria stubs demonstrated a mean increase of 0.0318 in optical density compared to control; the optical densities for 1 stub, 2 stubs, 3 stubs, and 4 stubs of planaria were 0.290, 0.347, 0.394, and 0.368, respectively (Figure 6D).

Finally, we used the MTT assay to investigate planarian regeneration in response to 1 nM of RvD1. Optical densities of planaria incubated in RvD1 were normalized to baseline. The optical density of planaria incubated in RvD1 started at 1.428 (SEM +/- 0.5720) 0 days after surgery. Optical density peaked at 2.588 (SEM +/- 0.4246) 3 days post-surgery before returning to 1.428 (SEM +/- 0.2731) 7 days post-surgery. At 7 days post-surgery, the optical density of regenerating planaria was 2.588 (SEM +/- 0.379) (Figure 6E).

Figure 3. Water RvD1 levels in whole planaria 2- and 8-days post incubation with 17-HDHA. Water samples from planaria habitat water (1000 µl) were analyzed 2 days and 8 days post-incubation with 1 or 10 µM 17-HDHA. Each dot represents 1 planaria. Regenerating group represents 2 planaria (2 heads and 2 tails). Data is mean ± SEM. Student’s t-test was performed between Ctrl and experimental groups. *p<0.05.

Figure 4. Planaria Eye Scoring Index. Representative images of score levels. Eye borders are demarcated with white dotted lines. Score 1 (planaria image) shows a pointer for one of the eyes.
may not be exposed to 17-HDHA naturally. Additionally, is not directly linked to planarian regeneration as planarians could also be a byproduct of an enzymatic upregulation that enhanced in regenerating planarians, suggesting that resolvins planarians. This likely means that RvD1 biosynthesis is release of RvD1 in the water compared to intact, unwounded omega-3 precursor.

DHA are one step upstream of 17-HDHA and are the earliest production in planaria when supplemented with DHA, as 17-HDHA. Moreover, further studies can investigate RvD1 production of Resolvin D2 (RvD2), so it remains to be seen planarian regeneration. For example, we did not measure the reveal other lipid mediators that are critical in orchestrating HDHA, so a mass spectrometry-based approach could likely that a variety of other lipid mediators (besides RvD1) in planarian regeneration, specifically RvD1 and its precursor 17-HDHA in Dugesia dorotocephala.

In this study, we observed an increase in RvD1 levels in planarian water samples in whole (unwounded) planaria incubated in 17-HDHA, but no change in tissue RvD1 levels between control and 17-HDHA-supplemented group was observed. This finding is exciting because this is the first time that D. dorotocephala are shown to have the biosynthetic pathways to synthesize RvD1 from 17-HDHA. In mammals, it is hypothesized that 17-HDHA is converted into RvD1 through enzymatic epoxidation by lipoxygenases. Therefore, further investigation is warranted to delineate the exact biosynthetic machinery in D. dorotocephala resolin production. It is also likely that a variety of other lipid mediators (besides RvD1) are also biosynthesized by planaria through DHA and 17-HDHA, so a mass spectrometry-based approach could reveal other lipid mediators that are critical in orchestrating planarian regeneration. For example, we did not measure the production of Resolvin D2 (RvD2), so it remains to be seen if planaria can also produce RvD2 when supplemented with 17-HDHA. Moreover, further studies can investigate RvD1 production in planaria when supplemented with DHA, as DHA are one step upstream of 17-HDHA and are the earliest omega-3 precursor.

We observed that “regenerating” planarians had a higher release of RvD1 in the water compared to intact, unwounded planarians. This likely means that RvD1 biosynthesis is enhanced in regenerating planarians, suggesting that resolvins play a role in planarian regeneration. However, resolvins could also be a byproduct of an enzymatic upregulation that is not directly linked to planarian regeneration as planarians may not be exposed to 17-HDHA naturally. Additionally, we observed a decrease in RvD1 levels in planarian tissue samples, which showed increased RvD1 in surrounding water. This was surprising and could be the result of changes in RvD1 efflux or release from regenerating cells in the blastema. Investigating the precise pathway by which 17-HDHA and other lipid mediators diffuse through the planarian cells into their environment, how RvD1 is made and released into the environment, and which cells are responsible for RvD1 production, was beyond the scope of this study. Future studies requiring sophisticated microscopy and histological analyses can examine which genes, enzymes, and cell types are involved in resolin biosynthesis in the planarian body after injury. Our results from the modified MTT assay indicated a high concentration of proliferating cells at the planarian blastemas, and future investigations could potentially explore resolin production specifically within the planarian blastemas.

Irrespective of classifying the family or class of resolvins that could be produced by planarians (we only tested one species), one exciting future possibility is to alter regeneration in planarians that are exposed to water taken from DHA/17-HDHA-incubated planarians. This could further identify potential non-specific class effects of these molecules in regulating regeneration. In other words, autocrine regulation of mediators might influence planarian regeneration and could have implications in mammalian regeneration as well. Our study highlights an exciting possibility of mass production of bioactive lipids like resolvins from planarian. Currently, resolvins are produced by chemical synthesis. Just like having bacterial systems to produce biologics or antibodies for therapies, our system can be potentially improved for
mass production of resolvins by planarians (regenerating). Planaria were successful in synthesizing RvD1 in this study, and further studies can be performed to confirm whether planaria can be used for the mass production of resolvin drug therapeutics.

In our studies, the eye scoring index was used for scoring planarian regeneration. Even though all eye scores in this investigation were all performed by one person, there is still a slight margin of variability in this assay, making it a moderately semi-quantitative approach. However, studying the time point of eye formation does provide an interesting benchmark to score planarian regeneration, and hence we used this to measure regeneration. One caveat of eye scoring assay is the time of the day used for observing eye formation. Due to barriers in experimental logistics, continuous monitoring of eyes could not be performed, hence the exact time-point of when eye formation reached our pre-developed scoring landmarks could not be identified precisely. Because of this disadvantage, we utilized a biochemical assay (MTT) to further shed light on regeneration index. The MTT assay was the empirically quantitative assay used in this investigation to measure regeneration index. While imaging planaria incubated in MTT, we observed that formazan crystals formed throughout the planarian body, more so during late stages of regeneration (5 and 7 days post-surgery) rather than at blastemas as previously observed (2, 3, and 0 days post-surgery). Further investigation is needed to identify the exact cell types that are metabolically active in the planarian body post-surgery, which could be cells near the blastema or far away from theregenerating tissue. To our knowledge, we are the first to report a quantitative method of assaying planarian regeneration through MTT assay, which is widely used to analyze cell metabolism and toxicity in cancer biology. Further studies can also refine the modified MTT assay by titrating the MTT dilution (we used 1:10 according to the assay protocol), potentially examining the ideal concentrations, temperature, and environmental factors needed to enhance the sensitivity of the assay.

In a recent study, the investigators encapsulated aspirin-triggered RvD1 into a biodegradable biomaterial to investigate a model of sterile inflammation using local, sustained delivery of the drug (12). As expected, the scientists observed an increase in the accumulation of cell debris phagocytizing monocytes and macrophages, however they also observed a potent pro-angiogenic and vascular remodeling response of RvD1 in the tissue (12). This study provided a role of resolving lipid mediators in differentiation and regeneration. While our study only observed oral ingestion/diffusion of 17-HDHA and RvD1 on planaria, future studies can examine the role of RvD1 in regenerative biology.

METHODS
Planaria care and dissection
Planaria species Dugesia dorotocephala was purchased

Figure 6. Planaria Regeneration assay based on a modified MTT method. A) Dissected planaria head after incubation in MTT for 4 hours; B) Planaria tail after incubation in MTT for 4 hours; C) Planaria mid-section stub after incubation in MTT for 4 hours; D) MTT assay readout (OD 562nm for formazan crystals) of different sizes of whole planaria and different number of planaria mid-section stubs after incubation in MTT for 4 hours, n=1 for each group; E) MTT assay readout (OD 562nm for formazan crystals) time course of regenerating planaria (head and tail) post-surgery; n=3 per time-point. Data is Mean ± SEM. F) MTT assay readout (OD 562nm for formazan crystals) of planaria head and tails (each n = two heads and two tails) on Day 0 to Day 7 post-surgery. Day 0, n=9; Day 2, n=10; Day 3, n=10; Day 5, n=9; Day 7, n=13. Data is mean ± SEM.
from Carolina Biological Supply Company (Burlington, NC) and were placed in Arrowhead spring water (Nestle waters, North America). Planaria were fed egg yolk on a bi-weekly basis and water was exchanged every 3-4 days.

On the day of surgical dissection, a chosen number of planaria of equal or comparable size were first isolated into a separate container apart from the habitat. In all experiments, planaria were transported by plastic pipettes with blunt end of approximately 0.5 cm diameter. Planaria were dissected along their horizontal midline while free-swimming. We avoided dissecting them over cold surface, as they constricted from cold temperature making dissections difficult to perform with uniformity across the groups. Dissection was performed with a clean scalpel blade in one single strike/motion. After dissection, planaria heads were transferred back to a new habitat, while planaria tails were relocated to incubation in a 6 or 24-well plate in chosen control or experimental wells. For incubation of planaria in RvD1 or 17-HDHA-treated water in 6-well plates, we used approximately 500 µl – 4000 µl mineral water per well.

**Protein Assay**

We used the Bradford method, which uses Coomassie Dye for determining protein in planaria lysates. Planarians or regenerating planaria heads or tails were transferred into a glass dounce homogenizer, and lysates were prepared on ice using slow clockwise and anti-clockwise rotations. The lysates were then transferred to eppendorf tubes and saved at -20°C until the day of use for assays. The Protein Determination kit was purchased from Cayman Chemicals (Ann Arbor, MI), and kit instructions were followed according to the company protocol. Protein Determination Assay reagent and Protein Determination BSA standard were prepared through serial dilution with UltraPure water. Protein BSA standard and the sample were first put into a 96-well plate before Assay Determination Reagent was pipetted into each well. The plate was incubated at room temperature for 5 minutes before measuring the absorbance at 595nm two times. Based on the standard values, a standard protein assay curve was plotted.

**RvD1 ELISA**

The RvD1 ELISA assay was used to measure RvD1 production in planaria tissue and water samples. The RvD1 Elisa kit was purchased from Cayman Chemicals, and was used according to company protocol. After defrosting at room temperature, ELISA buffer and the wash buffer were prepared by using UltraPure water to dilute (1X) reagents needed for the assay. A set of 9 standards were created through serial dilution where the first standard was 2000 pg/ml and the last standard contained 3.3 pg/ml of provided RvD1. Samples were thawed and spun down in a centrifuge at 4000 rpm (1800g). Standards and samples (50 µl) were transferred to 96-well plate with anti-serum and tracer, which was then covered in a film before incubating at 4°C for 18 hours. After incubation, the plate was washed with wash buffer five times, incubated with Ellman’s reagent for 90 min, and absorbance measured at 405 nm. The standard values were used to plot a standard RvD1 Assay Curve.

**Eye scoring**

Regenerating planarians were transferred one at a time (using a transfer pipet with a cut tip) onto a plastic surface. A droplet of water was provided for the planaria to swim freely and comfortably. Planarians were observed until a clear image of the eye was confirmed by the observer. In all the experiments, only the first author of this paper made the observations and scored the eye score to avoid any bias. Planaria eyes were scored on scale of 0-4 (Figure 4) based on the size and shape of the planarian eyes.

**Modification of MTT assay**

Planarians were first sorted according to lengths before grouping and dissection. In most experiments, similar sized planarians were used across the groups. In some preliminary experiments, groups were size-matched with equal number of large/mid-size/small planarians. Planaria were cut laterally right above the pharynx, and both head and tail portions were transferred to a 6-well plate using a cut transfer pipet and incubated in 4 mL of spring water or Resolvin D1. After an incubation period (either 0,2,3,5, or 7 days), 3.8 mL water (with/without RvD1) was aspirated out of the plate. The plate was tilted on a 30° angle and kept stable in the angle with planarians freely swimming in the 200 µl pool of water on the side of each well. Then, 20 µl of MTT solution was added in each well (1:10 dilution), and the planarians were incubated for 4 hours. Following the incubation, planaria were visually observed under a stereo microscope for capturing images and were then incubated with 200 µl of SDS solution (provided in the kit) for 12 hours at room temperature. Afterwards, the contents of each well were spun down briefly with a tabletop microcentrifuge to collect liquid to the bottom of the tube, and the solution was transferred into a 96-well plate and absorbance was measured at 562 nm. For each experiment, we also had a control group where planaria were incubated with MTT for a minute and then lysed with SDS. These control group values were used as baseline values (no formazan crystals), which were subtracted from 4 hour-incubation samples, to measure actual formazan crystal formation.

*Note: Anuran Chatterjee is employed with AstraZeneca (Wilmington, DE) and AstraZeneca has not played any role in the study nor has provided any financial support for this study. Dr. Chatterjee conducted the study on weekends outside his work hours with AstraZeneca.*

**REFERENCES**


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